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PRINCIPAL INVESTIGATOR: Debasis Mondal, Ph.D.

CONTRACTING ORGANIZATION: Tulane University Medical Center
New Orleans, LA 70112

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Debasis Mondal

E-Mail: dmondal@tulane.edu

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14. ABSTRACT
Adipose stem cells (ASC) are promising tools for delivering anti-cancer genes; however, studies show that ASCs may also increase tumor growth. We isolated ASCs from both healthy donors (ASC-cont) and cancer patients (ASC-PC). Migration assays were carried out to compare their tumor-tropism, and migratory ASCs were selected as sub-lines (ASC-sel). The SDF-1/CXCR4 axis was crucial in tumor-site specific recruitment of ASC-PC and ASC-sel cells. Tumor-predilection and tumor-promoting were compared in these three ASC population and their surface markers analyzed. In mice bearing C4-2B tumor xenografts, we tested ASC recruitment to prostate tumors. Compared to ASC-cont, ASC-PC cells exhibited higher and ASC-sel cells showed the highest tumor predilection. The ASC-sel cells also showed altered morphology and surface marker expressions, and their cotransplantation significantly increased tumor growth. The tumor-recruited ASCs underwent adipogenic differentiation commitment, and expressed androgen metabolizing enzymes (AMEs) and secreted testosterone (T). This implicated the role of patient-derived ASCs in tumor growth and recurrence of castration resistant prostate cancers (CRPC). In conclusion, we propose that, although efficacious in tumor-site recruitment, the autologous ASCs will not be safe for retransplantation in patients. Therefore, we suggest that ASC-cont cells with enhanced tumor-tropism but low tumor-promoting potential is identified, enriched and exploited as anti-cancer gene delivery vehicles.

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Prostate Cancer, Adipose Stem Cells, Invasion, Migration, Characterization, Lentiviral transduction, Tumor-homing, Tumor-growth, selection, In vitro, In vivo

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Introduction:

Several publications have shown clear evidence of tumor-site specific localization of Adipose stem cells (ASC) [1-4]. Therefore, tumor-homing potential of ASCs are promising tools for delivering anti-cancer genes to metastatic sites. However, studies have documented that only a small percentage of the systemically injected ASCs are actually recruited to the tumor site. Furthermore, when cotransplanted with tumor cells *in vivo*, studies also showed that certain ASCs may increase tumor growth [5-8]. Thus, identification of the tumor-promoting phenotype in ASCs, and a selection strategy to enrich ASCs with non-tumor-promoting activity but enhanced tumor-homing potential, will be crucial [9, 10]

The first objective (*Task-1*) of our project was to investigate whether the tumor-homing potential of ASCs can be enhanced, by either the *in vitro* enrichment of those ASCs which possess higher tumor predilection [11-13]. We have optimized several *in vitro* strategies for isolation of ASCs, from both normal individuals (ASC^n) and from PCa patients (ASC^{pc}), with enhanced invasion and migration capacity towards tumor-derived factors.

The second objective (*Task-2*) was to investigate whether the tumor-tropic ASCs can be engineered to express a potent anti-cancer gene, e.g. the herpes simplex virus thymidine kinase (HSV-TK) [14-17]. The HSV-TK enzyme can phosphorylate ganciclovir (GCV), and thus activate its cytotoxic effects on tumors that recruit HSV-TK expressing ASCs. We have shown that *in vitro* coculture of HSV-TK transduced ASCs were able to sensitize the PCa cells to GCV-induced killing.

The third objective (*Task-3*) was to identify specific signaling cues that are upregulated in ASCs following their recruitment to the tumor foci [18-21]. These signaling cues (transcription factor inductions) can be taken advantage of in order to augment the expression of HSV-TK, thus enabling localized GCV-induced cytotoxicity [9, 10]. We have shown that tumor-tropic ASCs undergo faster adipogenic differentiation and upregulate PPAR-γ and C/EBP-α transcription factors.

Ultimately, our fourth and final objective (*Task-4*) was to demonstrate that in PCa tumor xenografts *in vivo*, the ASCs colocalize with tumors and tumor-site specific HSV-TK expression is a successful anti-tumor strategy. We have been successful in showing tumor-site specific localization of the GFP-labeled ASCs. However, the cotransplantation of patient-derived ASCs (ASC^{pc} cells), but not those from normal donors (ASC^n cells) showed significant increase in tumor growth. We also observed that these engrafted ASC^{pc} cells underwent a tumor-mimicry, neoplastic transformation and released factors, such as androgens.

Studies with the *in vitro* enriched ASCs, clearly implicated that approaches that activate the invasive phenotype in ASCs and enrichment of those ASCs with tumor-homing ability, would facilitate the development of an optimal anti-cancer gene delivery vehicle. In the enriched subpopulations that have tumor predilection, both ASC^n and ASC^{pc}, we have identified surface markers that are associated with their invasive phenotype. (a Manuscript is *In Preparation* & a Patent has been filed; 61/516,671).

Interestingly, unlike the ASC^n cells, the ASC^{pc} cells have higher tumor-homing potential. We observed that when stimulated with conditioned media (CM) from PCa cells *in vitro*, these tumor-tropic ASC^{pc} cells were able to synthesize androgen-metabolizing enzymes (AME) and produced androgens in sufficient quantities to increase PCa cell growth. *In vivo* co-transplantation studies in mouse xenografts revealed that ASC^{pc} cells promoted PCa tumor growth and the tumor recruited ASC^{pc} cells expressed both androgen receptor (AR) and prostate specific antigen (PSA). Together, our results implicated a novel role for soluble factors from PCa cells in promoting intracrine androgen synthesis by the tumor-tropic ASCs. These critical findings with patient derived ASCs may lead to new developments in understanding the aggressive tumor growth in obese PCa patients. Taken together, our findings suggest that the mitogenic effects of patient-derived (autologous) ASCs will be a drawback in their clinical utility. We propose that although efficacious in tumor-site recruitment, the autologous ASCs will not be safe for retransplantation in patients, and we suggest that ASC^n cells with enhanced tumor-tropism but low tumor-promoting potential will be more efficacious gene delivery vehicles. Our findings will facilitate the identification, enrichment and exploitation of such enriched population of ASCs towards anti-cancer gene delivery.

Body:

(1). Adipose stem cell isolation and culturing. According to our previous published protocols [22, 23] we have isolated abdomen lipoaspirates from both Caucasian Americans (CA) and African American (AA) males, ages 25-60 yrs and body mass index (BMI) of 26.56 to 33.80. The ASCs obtained from normal individuals are designated as ASC^n cells. We also obtained abdominal lipoaspirates from PCa patients undergoing radical prostatectomy, and these lines are designated as ASC^{pc} cells.

Briefly, fresh fat tissue (~1 gm) were collected, washed three times in PBS, minced on ice into ~1 mm3 pieces. The minced tissue was suspended in 2 mg/ml of collagenase type-I (Gibco, Invitrogen, Carlsbad, CA) dissolved in PBS containing 5 mM calcium chloride and subsequently incubated at 37°C in a shaking water bath for 2 hr. To remove tissue debris, the cell suspension was successively filtered through 70 µm and 40 µm cell strainer (BD Biosciences, MD). Mature adipocytes were removed by centrifugation (1,500xg for 10 min) flowed by washing in PBS. The resulting stromal vascular fraction (SVF) pellet was resuspended and incubated for 2 min in a 2 ml lysis solution (0.15 M Ammonium chloride, 10mM Potassium bicarbonate and 0.1 mM EDTA) and centrifuged to remove RBCs. Cell pellets were washed in 2 ml 1% BSA (Sigma-Aldrich, MO), resuspended in DMEM/F12 medium (1:1; v/v) supplemented with 10% FBS and 1% antibiotics-antimycotic solution (penicillin G, streptomycin and amphotericin B; Mediatech, Herndon, VA) and maintained at 37^oC with 5% CO2.

The ASC^n cells were generously provided by Dr. Jeffery Gimble (Pennington Biomedical Research Center, Baton Rouge, LA). We had initiated our studies in four ASC^n stocks, which were characterized for their stem cell surface markers and monitored their differentiation potential towards adipocytes, chondrocytes or osteoblasts (Table-1). From 300 ml of lipoaspirates we routinely isolated up to 10^7 ASCs with greater than 95% purity. However, yields can vary widely between donors, and differences in both proliferation and differentiation potentials were also observed. Using these four batches of AT-MSCs, we have carried out invasion and migration assays. The specifics on these four ASC^n batches are given below:-

Table-1: Characteristics of ASCs from normal donors (ASC^n) used in this study.

Number	Passage	CD29	CD105	CD45	CD34	CD44	CD73	CD90	PE Ctrl	FITC Ctrl	PBS Ctrl
AT-MSC-01	P-0	98.6	97.10	4.3	89.25	5.25	93.55	85.65	3.59	1.34	2.1

Race: Caucasian Gender: Male **Age: 34** Height: 5 ft. 7 inches

Weight: 196 lb **BMI= 27.25** Depot: Abdomen Lipoaspirate

Number	Passage	CD29	CD105	CD45	CD34	CD44	CD73	CD90	PE Ctrl	FITC Ctrl	PBS Ctrl
AT-MSC-03	P-0	99.5	99.15	4.21	84.32	12.26	58.1	89.9	2.65	2.48	2.14

Race: Caucasian Gender: Male **Age: 18** Height: 5 ft. 10 inches

Weight: 185 lb **BMI= 26.56** Depot: Abdomen Lipoaspirate

Number	Passage	CD29	CD105	CD45	CD34	CD44	CD73	CD90	PE Ctrl	FITC Ctrl	PBS Ctrl
AT-MSC-04	P-0	99.23	98.41	4.39	89.51	5.12	93.45	85.45	2.57	1.56	1.89

Race: Caucasian Gender: Male **Age: 46** Height: 5 ft. 9 inches

Weight: 212 lb **BMI= 31.8** Depot: Abdomen Lipoaspirate

Number	Passage	CD29	CD105	CD45	CD34	CD44	CD73	CD90	PE Ctrl	FITC Ctrl	PBS Ctrl
AT-MSC-07	P-0	99.56	99.61	4.9	88.55	5.42	90.85	83.05	2.53	1.23	1.9

Race: Caucasian Gender: Male **Age: 55** Height: 5 ft. 8 inches

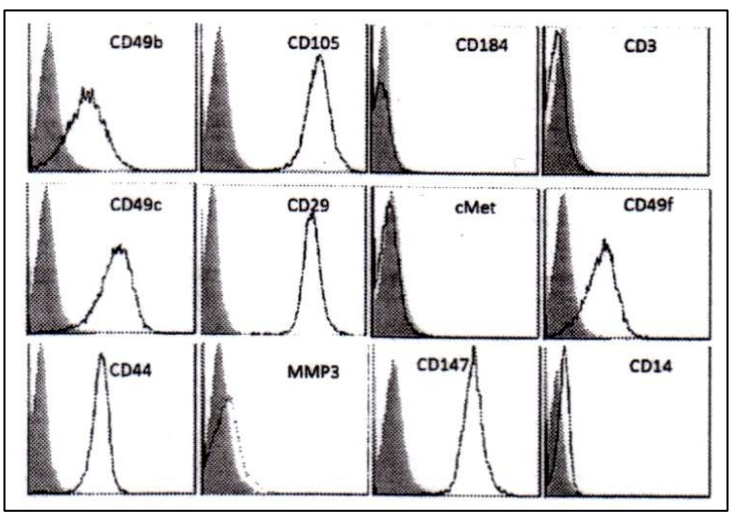
Weight: 222 lb **BMI= 33.8** Depot: Abdomen Lipoaspirate

(2). Fat tissue collection from patients. All information on research subjects was confidential and the study was conducted in accordance with an approved by an IRB protocol. Upon signing informed consent forms intra-abdominal lipoaspirates were procured from the space of *Retrius* near the dome of the bladder from PCa patients undergoing radical prostatectomy at Tulane University Hospital and Clinic, New Orleans, LA. Fat tissue was collected from at least 15 African Americans (AA) and Caucasian Americans (CA) prostate cancer patients with a mean preoperative PSA of 10.5 ng/ml , average age of 59.5 years, average Gleason score of 7.2 and average BMI of 32.4 (Table-2).

<u>Table-2</u>: Demographics and characteristics of ASCs from PCa patients (ASC^{pc}).

	pASCs	Race	Preoperative PSA	Age	Gleason Score	BMI
1	0157	AA	7.1	58	7	30.6
2	0455	AA	7.6	63	9	32.9
3	3866	CA	6.4	53	6	24.3
4	1011	CA	7.6	61	7	27.4
5	3035	AA	15.0	58	7	23.7
6	6062	CA	6.3	55	7	50.9
7	7777	CA	4.6	58	7	31.1
8	3724	AA	14.8	55	9	57.8
9	B123	CA	8.6	67	6	26.9
10	5470	AA	17.3	56	8	NA
11	5560	CA	4.8	62	6	24.7
12	7834	CA	4.7	61	7	29.2
13	3385	AA	7.0	67	9	30.4
14	A122	AA	14.2	53	6	34.7
15	5220	AA	31.4	66	7	28.4
Average ± SD (Min/Max)			10.5 ± 7.2 (4.6/31.4)	59.5 ± 4.8 (53/67)	7.2 ± 1.1 (6/9)	32.4 ± 9.9 (23.7/57.8)

(3). Flow Cytometry analysis of different ASC batches. The purity of isolated ASCs was verified by flowcytometry analysis as we described [24]. Briefly, cells (2 x10^6) were aliquoted, resuspended in 1 ml of PBS and incubated in the dark for 20 min at room temperature with one of the following antibodies: CD44-APC and CD29-peCy5 (BD Biosciences, MD), CD90-Pe-Cy5, CD105-PE, CD34-PE, CD45-PeCy7, CD79a-PeCy5 and CD11b-peCy5 (Beckman Coulter, CA). One cell aliquot was used as isotype control IgG1/IgG2a and another was unstained. Briefly, cells were initially stained with CD44-APC antibody (BD Bioscience, MD) for 15 min. After washing in PBS, the cells were then permeabilized by Intraprep permeabilization reagent according to the manufacturer's instructions (Bechman Coulter, CA). After washing, cells were stained with pan-cytokeratin-PE (C11) antibody (Cell signaling Technology Inc., MA) for 15 min. All cells were washed thrice by PBS and resuspended in 0.5 ml PBS, gently vortexed, and then analyzed by Beckman-Coulter Galios 2 Laser, 8 channel flow cytometer running Galios software for acquisition (Center for Stem Cell Research and Regenerative Medicine, Tulane University). A representative flowcytometry data is shown in **<u>Figure.1</u>**.



(4). Lentivirus transduction of ASCs and analysis of transgene expression.

Using published protocols for MSC transduction by our collaborators, Drs. Bunnell and Reiser, we have been able to optimize the protocol for lentiviral (LV) transduction in ASCs, by using polyethylenimine and generation a LV packaged in vesicular stomatitis virus glycoprotein (VSVg) [25, 26]. From the local 'Vector Core' facilities, we have obtained high titers (MOI >10^8/ml) of this LV which express green fluorescent protein (GFP) under the control of cytomegalovirus (CMV) promoter. Briefly, the LV was generated by using three-plasmids [550 µg of each of the lentivirus plasmid (pCMV-GFP); GAG and POL expression plasmid (pCMV-R8.74) and VSVg expression plasmid (pMD2.G)] which were transfected into 293T cells. Virus was concentrated by ultracentrifugation at 25,000 rpm for 90 minutes at 4°C. Viral concentrates were titered against 293T cells to establish the multiplicity of infection (MOI). For all experiments, ASCs were transduced within

the first three passages following initial plating and at less than 30-40% confluence. Cells were exposed to 10-50 MOI of the virus for 16 hours, thoroughly rinsed with PBS and returned to basal media. At 48 hours following transduction, cells were analyzed by flow cytometry (**Fig. 2a & 2b**) and immunofluorescence microscopy for transduction efficiency (**Fig. 2c**). Cells were cultured for 1-week and the high GFP-expressors were sorted by FACSorting (Fig. 2d). These cells maintained high level of GFP-fluorescence for at least 4-6 weeks in culture. Cryogenic stocks of these cells have been generated. High efficiency of lentivirus transduction with LV-GFP was achieved in almost all ASC stocks. A lower amount of cells stably expressed the reporter gene (GFP) for at least one month following transduction. The cryogenically stored transduced cells also showed stable expression of GFP following their sub-culturing.

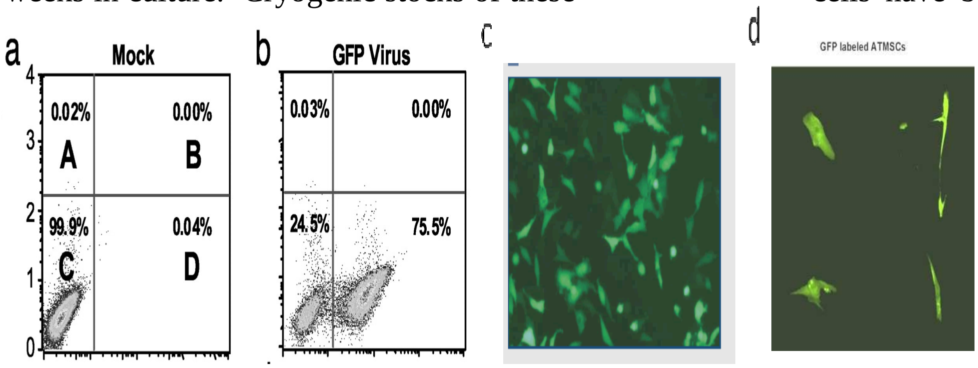


Figure-2: The GFP specific flow-cytometry (a & b) and fluorescence microscopy (c) data of a representative transduction in ASC (#07) is shown. Cells that retained GFP-fluoresence after 1-week were FACSorted for high GFP expressors (d) for propagation and freezing.

(5). PCa cell culture and preparation of conditioned medium (CM). The non-malignant prostate cells RWPE-1, androgen independent PC-3 cells and the androgen dependent LNCaP cells were obtained from American Type Culture Collection (Manassas, VA). The LNCaP isogenic androgen-independent C4-2B subline was a generous gift from Dr. L.W. Chung (Emory University, Atlanta, GA). RWPE-1 cells were cultured in keratinocyte serum-free medium supplemented with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract (Invitrogen Life Technologies, MD). LNCaP, C4-2B and PC-3 cells were cultured in RPMI-1640 medium (ATCC) supplemented with heat-inactivated 10% FBS and 1% penicillin/streptomycin (Invitrogen Life Technologies, MD). Cells were maintained at 37^{o}C in an air incubator with 5% CO2.

(6). Enrichment of tumor-tropic ASCs using trans-well culture chambers. The ASC sub-populations with high tropism towards bone metastatic PCa cells (C4-2B and PC-3) were enriched using an *in vitro* trans-endothelial migration (TEM) system (**Fig.3**), and the surface markers which are differentially expressed in migratory ASCs were analyzed by flowcytometry and real-time RT-PCR [27-32]. Using this TEM system, the differential migration of both ASC^{pc} and ASC^{n} cells towards CM of PCa was investigated. In another set of experiments, the adhesion and invasion ability of Calcein-AM stained PCa cells (1 x 10^{5}) towards CM of ASC^{n} and ASC^{pc} was compared in a TEM system for 2 hr and 24 hr. For both these studies, the human bone marrow endothelial cells (hBMEC-1) barrier (kindly provided by Dr. Graça D. Almeida-Porada, Univ. of Nevada, Reno, NV) cultured onto Matrigel-coated membrane inserts (8 µm pore size) in 12-well plates to generate a confluent hBMEC-1 barrier on the upper chamber. The CM from different PCa cells was then added to the lower chamber and different ASCs (1 x 10^{5}) were added onto the trans-well barrier and allowed to migrate towards the CM in the lower chamber for 48 hr. The ASC isolates with tropism towards PC cells were then propagated, stored and used (<passage-5) in subsequent experiments, and their migration, tumor-tropism and tumor-promoting effects were compared with the parental (non-enriched) ASCs.

The tumor-tropic ASCs were then transduced with a lentivirus construct (LentifectTM) expressing a cytomegalovirus (CMV)-driven green fluorescent protein (pLV-eGFP) according to the manufacturer's protocol (GeneCopoeia Rockville, MD). All experiments were performed in cells cultured in quadruplicates. Calcein-AM and eGFP fluorescence was measured at 485/528 nm and 485/520 nm, respectively, by a fluorescence microplate reader (BIO-TEK Instruments, Winooski, VT) and data was expressed as Mean ± SE of three independent experiments as we described [33]. Conditioned medium (CM) from normal (RWPE1) or PCa (LNCaP, C4-2B and PC-3) cells were prepared by washing cells (80% confluent) three times in DPBS followed by their cultured in phenol red-free, serum-free RPMI-1640 for 24 hr. The CM was then collected, filtered (0.2 µm) and stored at -20^{o}C until used. The CM from both ASC^{n} and ASC^{pc} cells was also obtained from 70%

confluent ASC cultures, filtered (0.2 µM) and stored at -20°C until used. For specific experiments, the CM from both ASCs and PCa cells were collected in a medium supplemented with 10% charcoal-stripped FCS.

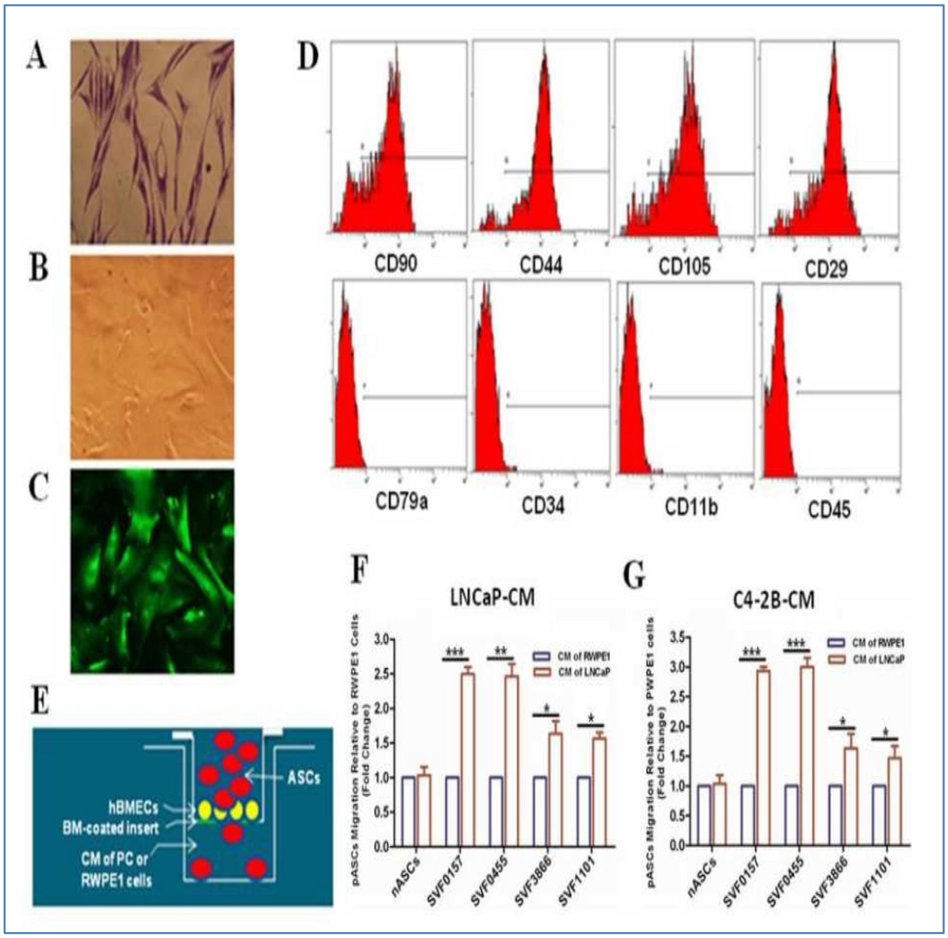


Figure-3. Isolation, characterization and TEM of ASC^n and ASC^{pc} cells. Representative photomicrographs (40x) of methylene blue stained ASC^{pc} isolates, demonstrated retention of fibroblast-like phenotype (A); ASCs under bright field (B); ASCs stably transduced with pLV-eGFP (C). In (D), flowcytometry of CD29, CD44, CD90, and CD105 (upper panel) and hematopoietic lineage markers CD11b, CD34, CD45, and CD79α (lower panel) are shown. In (E), a schematic illustration of the trans-endothelial migration (TEM) system used for migration and invasion analysis, is shown. In (F) & (G), migration of pLV-eGFP-labeled ASC^n or ASC^{pc} [designated here as serum vascular fractions (SVF)], are shown. Migration was measured using a fluorescence plate reader. Migration in quadruple is expressed as a fold change in fluorescence intensity of ASC^{pc} cells after normalization to that observed with the ACS^n cells. (n=3); *, ** and *** denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

These findings clearly showed that the patient-derived ASCs migrate faster towards tumor-derived factors (CM), suggesting that the enrichment of these cells may prove to be a superior delivery vehicle. We wanted to see if this phenotype can be identified or induced in the normal ASC^n cells, as well.

In four different normal ASC batches (01, 03, 04 and 07) we carried out *in vitro* migration and invasion assays. In order to identify those cells which can efficiently traverse membrane barriers and those which actively invade through extracellular matrix (ECM), both migration (**Fig. 4A**) and invasion assays (**Fig. 4B**),

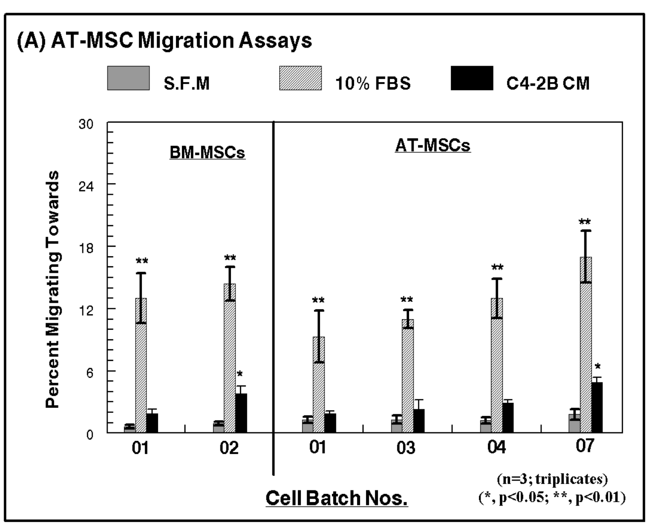


Fig. 4A

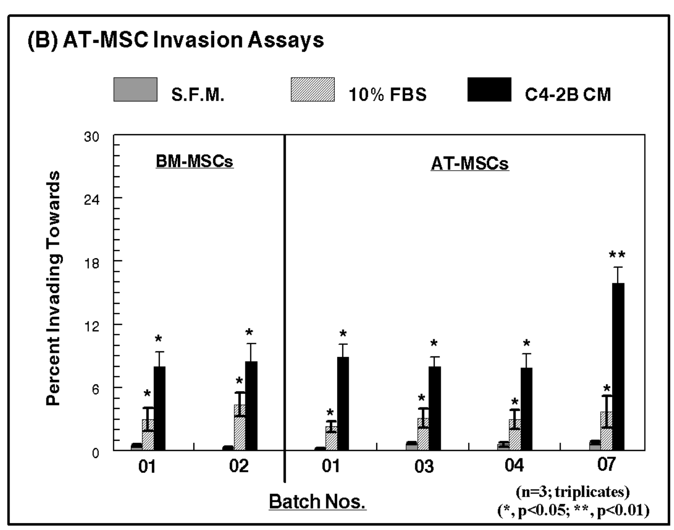
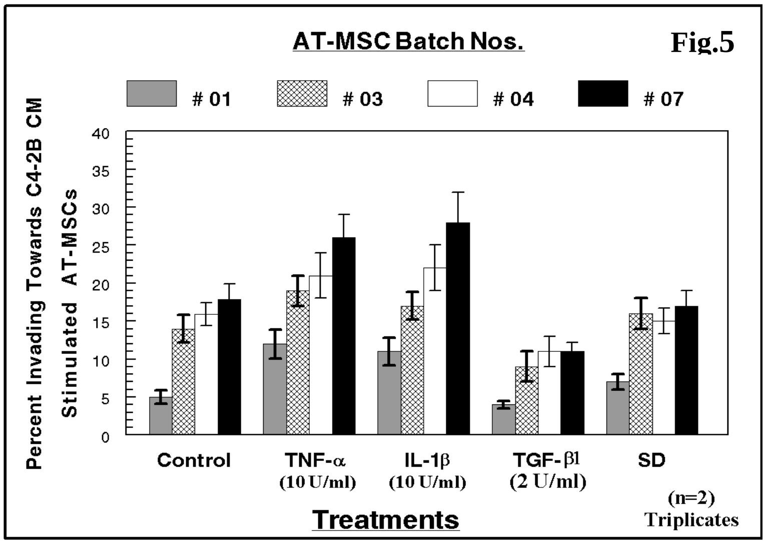


Fig. 4B

were carried out, towards either serum free medium (S.F.M.), medium containing 10% FBS, or towards tumor-derived factors (C4-2B conditioned medium). In the migration assays, we measured percentage of cells traversing (after 6 hrs) via the transwell membrane insert only (8 μ pore size) and for the invasion assays, percentage of cells travelling through a matrigel barrier followed by migration via the transwell membrane insert, were determined at 16 hrs. These two different assays were carried out to critically demonstrate that certain ASC^n cells have active migration and invasion ability towards tumor-derived factors, which requires the actions of cell adhesion molecules (CAMs), integrins and matrix metallo-proteases (MMPs), rather than simple migration towards a concentration gradient of growth factors and/or chemokines.

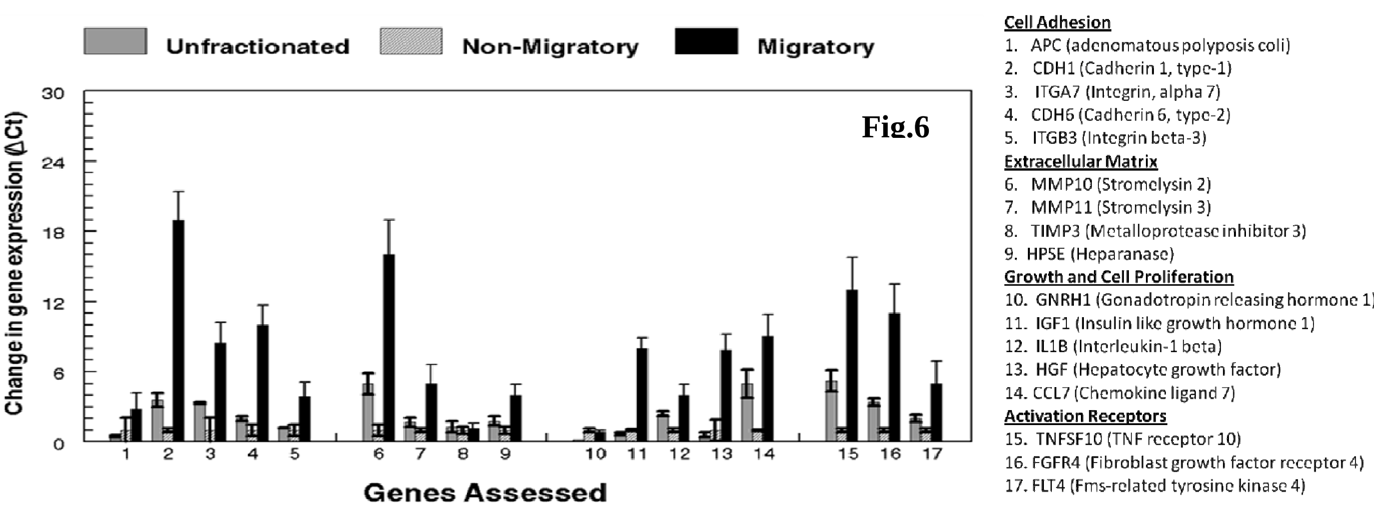


Next, we carried out further studies in normal ASCs in order to determine whether *in vitro* manipulation of ASC^n cells can be carried out to enhance their migratory and invasive abilities towards tumor cells (**Fig. 5**). These trans-well culture studies were carried out using ASCs which were stimulated for 2 hrs with either TNF-α (10 U/ml), IL-1β (10 U/ml) or TGF-β1 (2 U/ml), following which they were washed and then cultured for 16 hrs before labeling with Calcein-AM and use in trans-well invasion assays. The migrations of unstimulated (control) or stimulated ASCs towards C4-2B CM were analyzed at 16 hrs and percent of cells in the lower *vs* upper chambers were calculated.

Results: A percentage of the ASC^n cells have tumor homing properties *in vitro* which were much lower than that observed with ASC^{pc} cells. However, stimulation of ASC^n cells increased their migration and invasion capacities. Specific migrations towards tumor-derived CM were only observed in the matrigel invasion assays and not in their migration towards 10% FBS. In the absence of matrigel, more cells migrated towards 10% FBS and less towards tumor-CM. We further observed that, as compared to either LNCaP or RWPE-1 cell derived CM, tumor-tropism of ASCs was especially higher towards the aggressive prostate tumor cell lines, e.g. C4-2B and PC-3. These studies enabled us to enrich for ASC clones, for the following *in vitro* and *in vivo* studies.

(7). Differential marker expression in migratory vs. non-migratory ASCs.

From the *in vitro* studies we have isolated several clones of ASCs that possess enhanced migratory ability. These cells were propagated and aliquots of cells have been used for further studies to monitor differential expression of genes. Expression of genes which are closely associated with cell adhesion and migration, e.g. integrins (Int), cadherins (Cdh) and matrix metallo-proteinases (MMPs) were compared in the unfractionated (total), non-migratory and the migratory ASCs (**Fig. 6**).



The expression of markers were determined by *Real-time* RT-PCR assays and fold changes in expression of specific genes were determined by using the RT2 Profiler custom PCR array (SuperArray Biosciences, # PAHS-028D). Briefly, in a 96-well plate SuperArray system we simultaneously examined the mRNA levels of 89 genes including five ‘housekeeping genes’, according to the Manufacturer’s protocols. The first-strand cDNAs were synthesized from 2.5 µg of total RNA and quantitative PCRs were performed independently for each subpopulation obtained from AT-MSC (cell clone #03). Values obtained for the threshold cycle (Ct) for each gene were normalized using the average of four house-keeping genes (HPRT1, RPL13A, GAPDH, ACTB). Analyses were performed using the software provided by the manufacturer, and the fold change (ΔΔCt) between the unfractionated, non-migratory and the migratory populations were assessed. The genes which showed highest differences in expression between the subpopulations are shown.

Results: The migratory subpopulation expressed significantly higher levels of Cdh-1, Cdh-6, Int-α7, HGF, IGF-1, both TNF and FGF receptors, as well as both MMP-10 (stromelysin-2) and MMP-11 (stromelysin-3).

(8). ASC migration occurs more rapidly towards PCa cells seeded within bone marrow stromal cultures.

In order to identify whether tumor-associated stroma also releases factors that enhance ASC migration, we carried out studies using CM from of PCa cells (C4-2B) alone, or with CM from cocultures with bone-marrow stromal cells (BMSCs), either control or osteogenically stimulated cells (**Fig.7**). For these transmigration studies, ASC^{n} cells from three different donors which had shown varying degrees of migration in our previous studies. These were ASCs from PCa patients, an African American (AA) or Caucasian American (CA), and one from a normal donor. These lines are designated as D1 (ASC^{AA}), D2 (ASC^{CA}) and D3 (ASC^{N}). The differences in percent migration, as compared to total number of cells added to the top chamber of the TWCs, are shown in the bar graphs. We observed significant differences in the migratory phenotype of different ASCs (D1, D2 & D3) towards CMs from either C4-2B or BMSCs alone, or towards CM from C4-2B-BMSC cocultures.

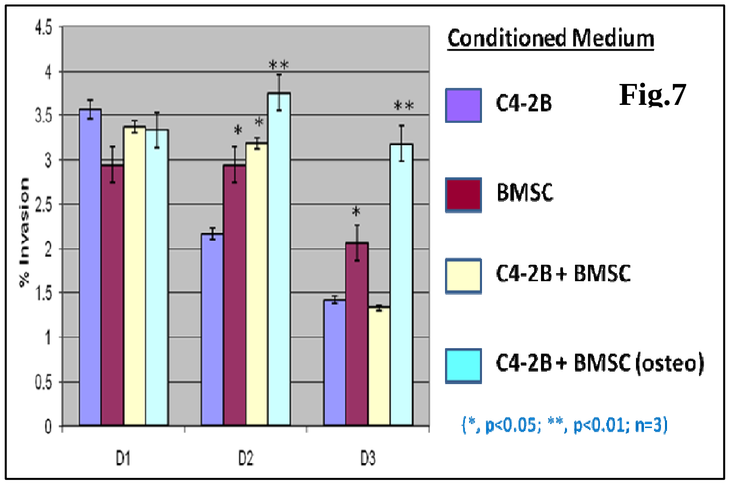


Fig.7

These observations clearly suggested that factors secreted by the tumor-cells as well as from the tumor-associated stromal cells increase tumor-site specific recruitment of ASCs. Interestingly, the ASC^{AA} cells showed high rate of migration to CM from C4-2B cells which was not significantly increased when CM from PCa-BMSC cocultures were used. However, both ASC^{CA} and ASC^{N} cells showed higher rates of migration towards CM from PCa-BMSC cocultures. Furthermore, the highest level of migration in these cells was clearly evident when CM from the osteogenically stimulated C4-2B + BMSC cocultures was used. These findings may elucidate why PCa cells primarily metastasizes to osteoblastic areas of the bone marrow.

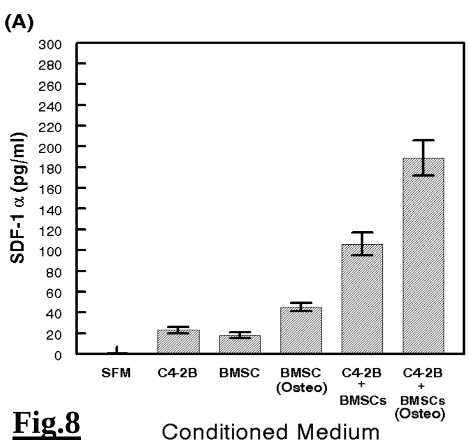
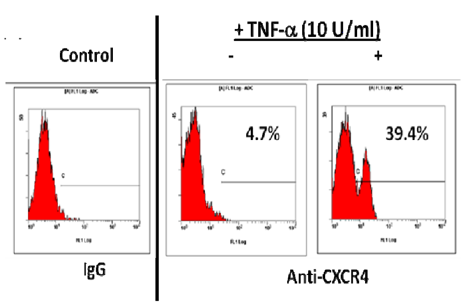


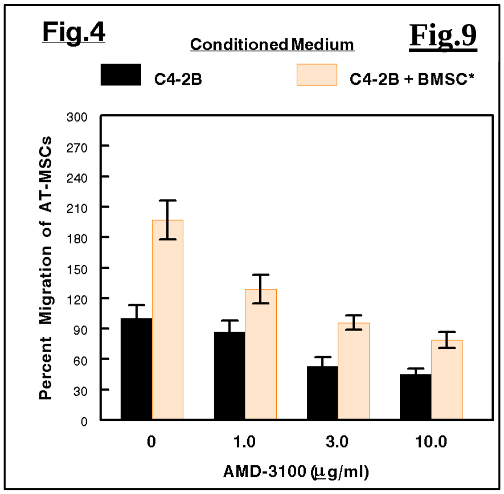
Fig.8



(9). Crucial role of SDF-1α/CXCR4 axis in ASC migration towards PCa-BMSC cocultures.

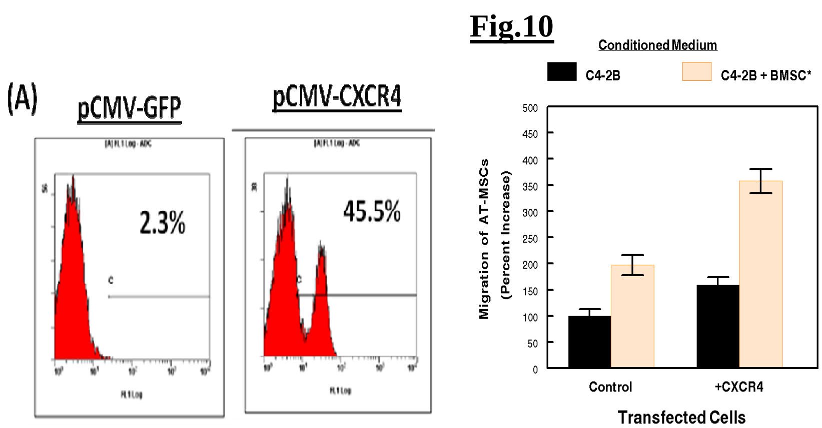
The enhanced migration to tumor-stroma may utilize attraction towards chemokine gradients [34-36]. Since osteoblasts secrete large quantities of the chemokine stromal derived factor-1 alpha (SDF-1α) we first measured SDF-1α levels in PCa-BMSC cocultures by using an SDF-1α specific ELISA assay (**Fig.8A**). Flowcytometry analyses were also carried out to determine the level of CXCR4 expression in both

unstimulated and TNF-α (10 U/ml) stimulated ASC^{n} cells (**Fig. 8B**). Our investigations showed that supernatants from both C4-2B cells and BMSCs had minimal levels of SDF-1α (30-70 pg/ml), however, the PCa-BMSC cocultures, especially the osteogenically stimulated cultures, showed significantly ($p<0.01$) higher level of SDF-1α production (100-200 pg/ml). In addition, flowcytometry analyses using a fluorescent (PE) conjugated antibody to CXCR4 suggested that a small subpopulation of the ASCs (<5%) expressed CXCR4 (receptor for SDF-1α) under unstimulated condition, however, a significant increase in surface CXCR4 expression 8-10 fold was observed in these ASCs following their TNF-α (10 U/ml) stimulation. This clearly indicated the crucial role of SDF-1α-CXCR4 chemokine axis in tumor-stroma specific homing and corroborated our hypothesis that ASCs possessing an activated phenotype can migrate better towards osteoblastic areas of the bone.



In order to determine whether the SDF-1α/CXCR4 axis is indeed involved in the enhanced ASC migration towards PCa-BMSC cocultures, we monitored the effects of a CXCR4 antagonist, AMD-3100 (**Fig.9**). We observed that pre-incubation of ASCs with AMD-3100 (1-10 µg/ml) significantly inhibited their migration towards PCa-BMSC conditioned medium.

Next, we hypothesized that since the SDF-1α/CXCR4 axis is crucial in ASC recruitment, then it may also be utilized to enhance ASC homing to tumor microenvironments expressing SDF-1α. In ASCs transiently transfected with a CXCR4 expression plasmid (pCMV-CXCR4), we observed significantly ($p<0.05$) increased surface levels of CXCR4 by flowcytometry analysis (**Fig.10A**). In addition, we observed that ASC migration towards CM from tumor-stroma cocultures could be enhanced by overexpression of CXCR4 (**Fig.10B**).



These observations clearly underscored the importance of SDF-1α released by the PCa cells and from the tumor-associated BMSCs. Our findings also demonstrated that the tumor-homing properties of ASCs, which may be largely dependent upon their expression of CXCR4, may be taken advantage of in future gene therapy strategies to increase tumor-site specific recruitment *in vivo*. (Manuscript *In Preparation* & Provisional Patent filed; # 61/516,671).

(10) ASCs expressing the HSV-TK enzyme enhanced GCV-induced killing on cocultured PCa cells.

The <u>second</u> objective of our proposal was to investigate whether the tumor-tropic ASCs can be engineered to express the herpes simplex virus thymidine kinase (HSV-TK) gene which can locally activate the cytotoxic effects of ganciclovir (GCV) following their recruitment to tumor sites. Although our transduction data was promising, we observed that the LV-transduced ASCs showed lower proliferative potential and had especially dampened differentiation capacities. This would be a significant deterrent to their utility in our future studies. Furthermore, LV integration in ASCs may also have untoward long-term side effects and would be problematic towards their clinical use in patients. Hence, we envisioned that since ASCs can localize to tumors within 2-3 days [1, 3, 4] where a transient expression of our prodrug therapy (HSV-TK/GCV) would be sufficient towards a potent anti-cancer effect, we looked for an alternative strategy for high efficiency gene delivery in these cells which would not affect their proliferative and differentiation capabilities.

(11). A plasmid based transfection of ASCs may be more efficacious in delivering of HSV-TK to tumors.

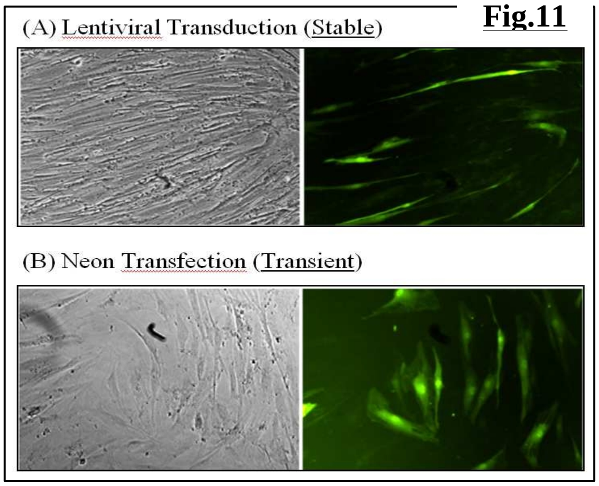


Fig.11

By using a Neon™ transfection system from Invitrogen (a high-efficiency and low toxicity electroporation technique) we have first optimized a transient transfection protocol with 0.5 μg of a plasmid encoding the EGFP in ASCs (~10 million cells). The following conditions were found to be optimal: Pulse voltage of 1400 volts, pulse width of 10 milliseconds and pulse number of 2-3. In **Fig.11,** we are presenting data comparing the efficiencies of the LV infection and Neon™ transfection protocols by using vectors expressing EGFP (reporter gene). In (A), transduction with VSVG pseudotyped LV was carried out, and in (B) transient transfection with the Neon system was carried out. The EGFP expression was checked after 5 days and photographed using a TS-100 nikon inverted microscope.

Our data showed that optimized *Neon™* transfection system show high efficiency of ASC transfection. This enabled almost 80-90% transfection efficiency in ASCs and showed less than 70% cytotoxicity. Furthermore, the transiently transfected cells were competent for both proliferation and lineage specific differentiation. In addition, the EGFP expression was seen to be stable for at least 10-12 days. Hence, we modified our approach from a stable to a more transient gene expression strategy using the plasmid based gene therapy approach.

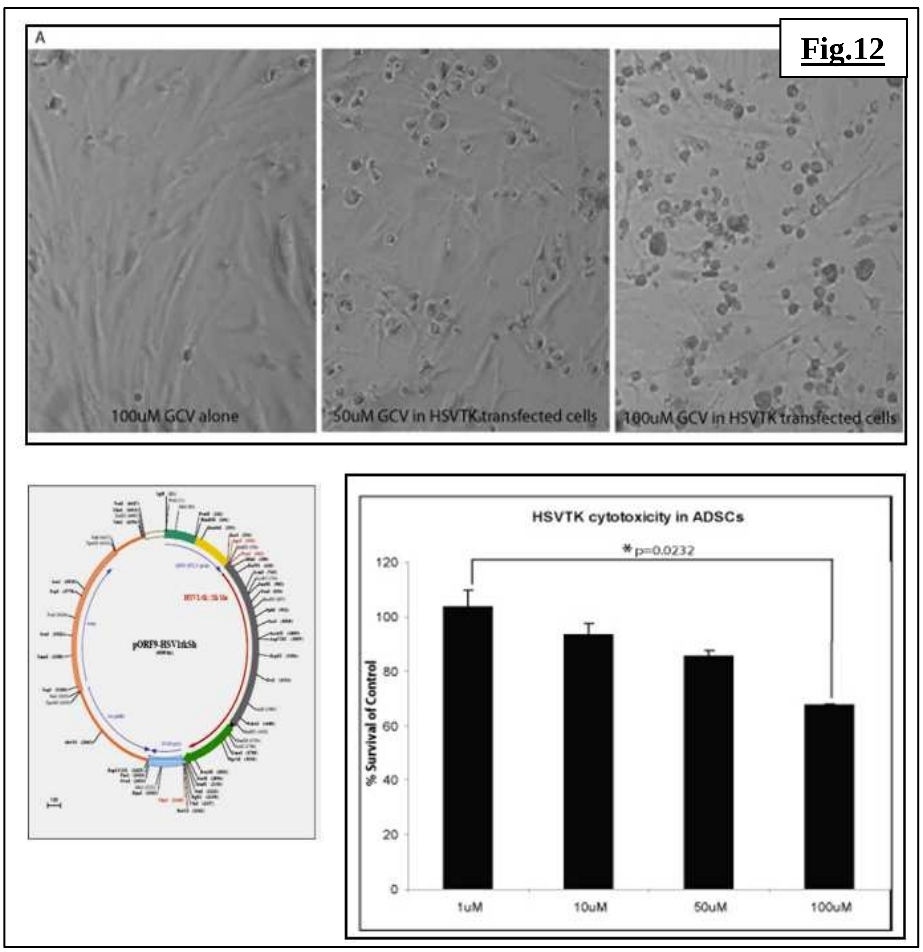


Fig.12

We used the *Neon™* transfection protocol to transfect ASCs with a HSV-TK expression plasmid (pORF-HSVtk; Invivogen). The plasmid pORF-HSVtk constitutively expresses the transgene under the control of a eukaryotic elongation factor (EIF-2α) enhancer [plasmid map in Fig.12, left]. Approximately 24 hrs post transfection, cells were exposed to different concentration of ganciclovir [GCV (1-100 μM) for 5 days. Exposure to the higher concentrations of GCV (50 & 100 μM) was significantly more cytotoxic (p=0.0232) and induced > 45% death in the HSV-TK transfected cells only (**Fig.12).** The light microscopy images show cell death and bar graphs showing percent survival following exposure to increasing concentrations of GCV. These results indicated that GCV treatment of cells transfected with HSV*tk* can be effective in *suicide gene therapy*. (Provisional Patent filed; # 61/516,671).

Next, we wanted to observe whether coculturing of the HSV-TK expressing ASCs with untransfected PCa cells would be able to show a *bystander killing effect* (**Fig. 13**) [37]. The ASCs expressing HSV-TK were cocultured at different dilutions with the C4-2B cells and percent cell viability in 5-days were measured by MTT assays. The ratios of C4-2B cells were varied with a constant number of transfected ASCs. Higher rate of killing was clearly evident when cells were plated in 1:1 and 1: 0.5 ratios of PCa cells to ASCs. As much as a

70-90% elimination of the PCa (C4-2B) cells was observed in this coculture experiments, thus clearly demonstrating a bystander killing effect. Hence, we have established that the *Neon*^{TM} transfection system can be used to express both CXCR4 and HSV-TK [37, 38].

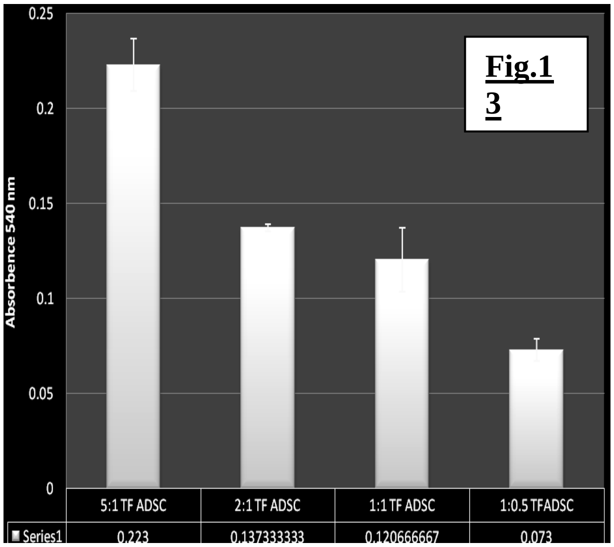


Fig.13

Interestingly, the GCV-induced cytotoxicity was far greater in the PCa cells (Fig. 13) as compared to the suicide killing effect observed in the AT-MSCs (Fig.12], further corroborating that the residual (surviving) AT-MSCs may have potential long-term anti-tumor effect following multiple GCV exposures *in vivo*. However, we also envision that even low level of ASC migration to systemic sites and the expression of HSV-TK at these nonspecific areas would have significant non-target toxicity from GCV, which will be unacceptable in a clinical setting. Hence, signaling cues will need to be in place in order to regulate the HSV-TK expression only following tumor-site specific recruitment of the engineered ASCs, which formed the basis of our Task-3.

(12). Tumor-derived factors increased both osteogenic and adipogenic differentiation of ASCs.

Our ultimate goal was to investigate whether we can utilize the lineage specific transcription factors in regulating HSV-TK expression from ASCs. Therefore, we first determined the effects of tumor-derived factors (C4-2B CM) on ASC differentiation towards either adipogenic or osteogenic lineages. Next, we have monitored the temporal expression of two lineage specific transcription factor genes, PPAR-γ (adipoenic) and RUNX2 (osteogenic) by RT-PCR [39-41].

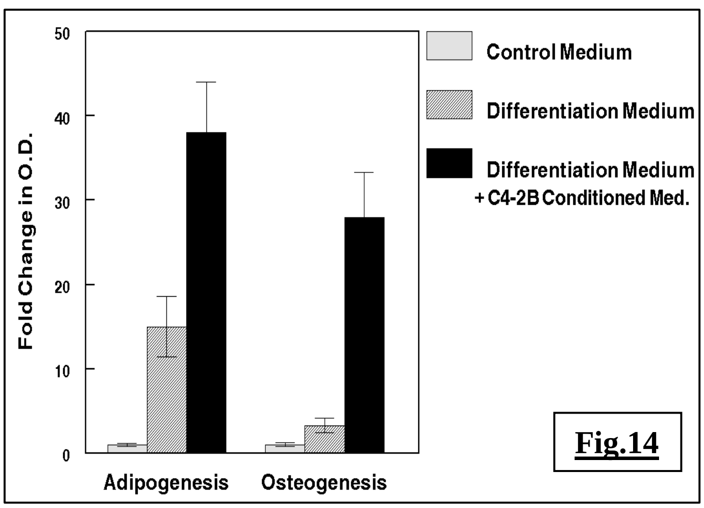


Fig.14

For <u>adipogenic differentiation</u>, cells were incubated in DMEM medium (0.5 µM 1-methyl-3 isobutylxanthine, 1µM dexamethasone, 10 µg/ml insulin and 100 µM indomethacin) for 3 weeks. To visualize the extent of lipid droplets, cells were fixed with 4% formalin and stained with Oil red-O dye. For <u>osteogenic differentiation</u>, cells were incubated in medium containing 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM L-ascorbic acid-2-phosphate for 3 weeks. Mineralization of the extracellular matrix was visualized by staining with Alizarin red dye. The extent of differentiation was measured colorimetrically by organic phase extraction (isopropanol) of dye and measuring optical density (OD). Our findings showed that tumor-derived factors can increase both osteogenic and adipogenic differentiation of ASCs (**Fig.14**). Our data clearly showed a more rapid increase in these differentiation markers in AT-MSCs exposed to tumor-derived factors.

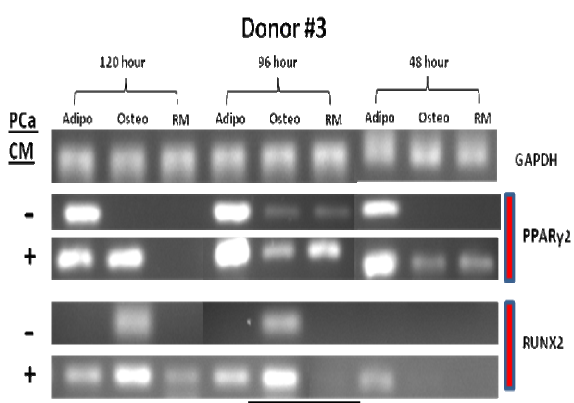


Fig.15

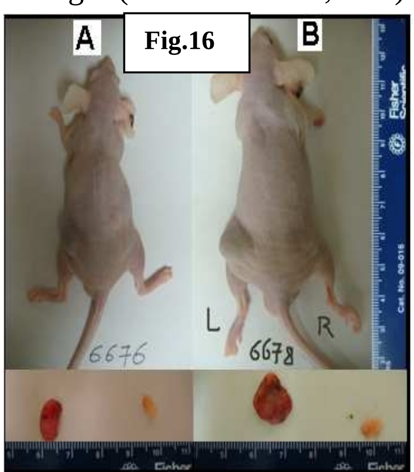
We then measured the mRNA levels for GAPDH, PPAR-γ and RUNX2 were measured every day following differentiation induction, for 7-8 days [**Fig. 15, next page**]. Following differentiation induction, the ASCs were exposed to either serum free medium (-) or conditioned medium from C4-2B cells (+). The most significant changes in control and tumor-exposed ASCs were observed between day-2 and day-5 (48-120 hrs) post differentiation induction, and results from these time points are included in the figure [**Fig. 15, left**]. In this representative ASC line, the lineage specific transcription factors were rapidly

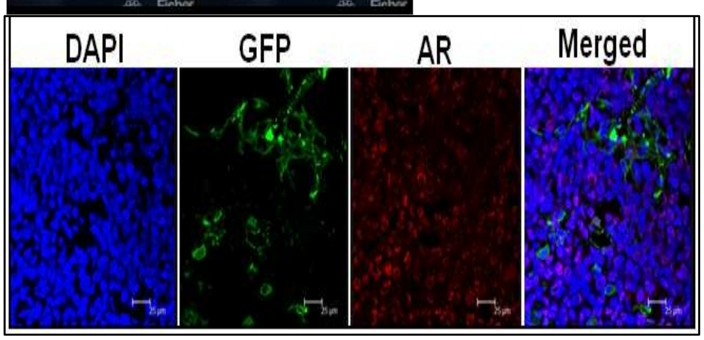
increased within 48 hrs post exposure to either adipogenic or osteogenic media. Similar to the enhanced differentiation observed in Fig. 11, the increase in both PPAR-γ and RUNX2 was found to be much higher in cells coexposed to the PCa-CM, as well. The induction of PPAR-γ (adipoenic) was more robust and more consistent, whereas expression of RUNX2 was weaker and its induction was seen in both adipogenic and osteogenic cells, especially at the later time point. From this data it became evident that the PPAR-γ response element may be used to effectively increase HSV-TK gene expression in tumor-recruited ASCs. By using this vector, we proposed that ASCs recruited to tumors will enhance their HSV-TK expression and GCV cytotoxicity *in vivo*. Therefore, we had initiated studies in PCa tumor xenografts to first determine the *in vivo* tumor-tropism of the enriched ASCs and the effects of tumor-recruited ASCs on PCa tumor growth *in vivo*.

(13). Tumor-tropic ASCs engraft in PCa tumors and promote tumor growth *in vivo*.

We developed PCa xenografts in immunocompromised mice (an IACUC approved protocol). Six-week-old athymic BALB/C nude (nu/nu) male mice (Harlan, Indianapolis, IN) were housed in a pathogen-free facility and were given free access to commercial rodent chow and water according to approved IACUC animal protocol at Tulane University. To assay for the ability of different ASCs to promote PC cell growth *in vivo* [42], the enriched ASC^n and ASC^{pc} cells were transduced with a pLV-eGFP and stable clones were selected with puromycin, expanded, and screened for GFP expression by Nikon E400 fluorescence microscopy (Nikon Instruments, Melville, NY).

To assess the effect of ASCs on PCa cell growth *in vivo*, C4-2B cells (1×10^6) mixed with eGFP-expressing ASC^n or ASC^{pc} cells (1×10^5) were suspended in 50 μL of serum-free DMEM medium and an equal volume of Matrigel (BD Bioscience, MD) and subsequently transplanted subcutaneously (s.c.) by a syringe fitted with a





27-gauge needle into the right and left flanks of each mouse (**Fig.16**). The control mice were injected with C4-2B, ASC^n or ASC^{pc} cells alone. Tumor formation was monitored up to 10 weeks. The tumor sizes were measured at different time points according to the following formula: 1/2 (Length × $Width^2$). The tumors were excised, weighed, and sectioned for H&E staining, IHC and immunofluorescence analysis. Part of the tumors was snap-frozen or paraffin-embedded for further analysis. The frozen sections were fixed in cold acetone/methanol (1/1 v/v) solution, washed thrice with PBS, blocked with 10% of goat serum for 1 hr after washing twice with 0.2% Triton X-100 in PBS, then incubated (4°C, 12–16 h) with primary antibodies (goat anti-AR, 1:100), rabbit anti-PSA (1:50), anti-rabbit α-methylacyl-CoA racemase (AMACR) (1:200), anti-rabbit anti SRD5A1 (1:100), mouse anti–cytokeratin 5/18 (CK5/18) (1:200), rabbit anti-cytokeratin 8 (CK8) (1:200), rabbit anti-Ki67(1:200; NOVUS Biologicals, Littleton, CO) as well as rabbit anti-Von Willebrand factor (1:200) (abcam) and mouse α-smooth muscle actin (1:500) (Sigma-Aldrich, St. Louis, MO). Tissue sections were then incubated for 1 hr at room temperature in PBS/0.05% Tween-20 solution containing secondary antibodies (1:500) including donkey anti-mouse, rabbit, and goat Alex Flour 488, 594, or 647-conjugated IgGs; (Molecular Probes, Invitrogen, Carlsbad, CA). Slides were mounted with mounting medium with DAPI to stain the nuclei (Vectashield, Burlingame, CA). Images were acquired by confocal Leica TCS SP5 microscope/LAS AF software or Olympus IX70 inverted fluorescence microscope/MagnaFire software.

The GFP-labeled ASCs, even though injected at a distal site showed tumor-site specific localization. In panel **(C)**, data from immunohistochemical (IHC) analysis of tumor sections with antibodies to androgen receptor (AR) and immuofluorescence microscopy (IFM) for GFP-labeled cells, are shown. DAPI stained nuclei are shown in blue. The engraftment of GFP-transduced ASCs (*green*) in C4-2B xenografts expressing AR (*red*) is clearly evident in the merged image. We also observed that the C4-2B tumors in ASC coinjected

mice were almost two times larger than those coinjected with CV-1 cells. Tumors generated in coinjected mice were grossly associated with increased neovasculatures, suggesting that ASCs may induce angiogenesis which can contribute to the increased PCa tumor mass. Although this may contradict the utility of using ASCs as anti-cancer gene delivery vehicles, we believe that our approach to locally activate the cytotoxic effects of GCV should be able to suppress the mitogenic effects of the tumor-tropic ASCs from patients. Therefore, we first wanted to understand the mechanism/s linked to the tumor-promoting effects of ASCs.

(14). Tumor recruited ASCs show increased androgen-metabolizing enzymes (AME) expression.

Adipocytes are known to secrete steroid hormones, e.g. estrogens and androgens, which may increase tumor growth. We examined the expression of androgen-metabolizing enzymes (AME) involved in the *de novo* biosynthesis of testosterone in both ASC^{n} and ASC^{pc} cells, under unstimulaed conditions and following their stimulation with PCa CM. Expression of 3-β-hydroxysteroid dehydrogenase (3-β-HSD), 17-β-hydroxysteroid dehydrogenase (17beta-HSD subtype 2, 3 & 5) were compared in both ASC^{n} cells and in ASC^{pc} cells (SVF0157 and SVF-455). For these studies, control medium (serum-free RPMI-1640) or CM (1:1; *v/v*) derived from PCa cells were added to ASC^{n}s or ASC^{pc}s (70 % confluent) which were pre-cultured in medium supplemented with 10% charcoal-stripped FBS 24 h, and subsequently harvested at various time frames (up to 96 hr). The expression of androgen-metabolizing enzymes (AME) in ASCs was analyzed by qRT-PCR [43] (**Fig. 17)**, and the androgen production in culture supernatants was measured by an ELISA kit (Cayman Chemicals, Ann Arbor, MI) (**Fig. 18)**. The results were normalized to controls and are expressed as fold change ± SE from three independent experiments.

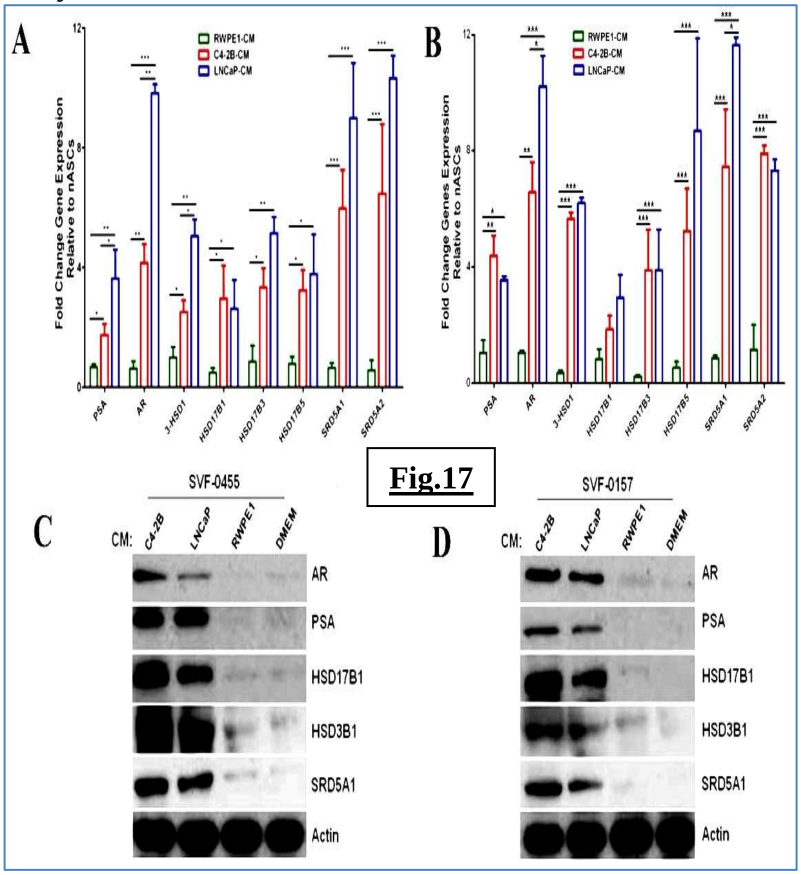


Fig.17

Exposure to factors secreted by the PCa cells conferred transcriptional upregulation of steroidogenic gene expression by the patient-derived ASCs *in vitro*. Interestingly, the CM-induced upregulation of AME transcripts was significantly higher in ASCs procured from African American than Caucasian American patients.

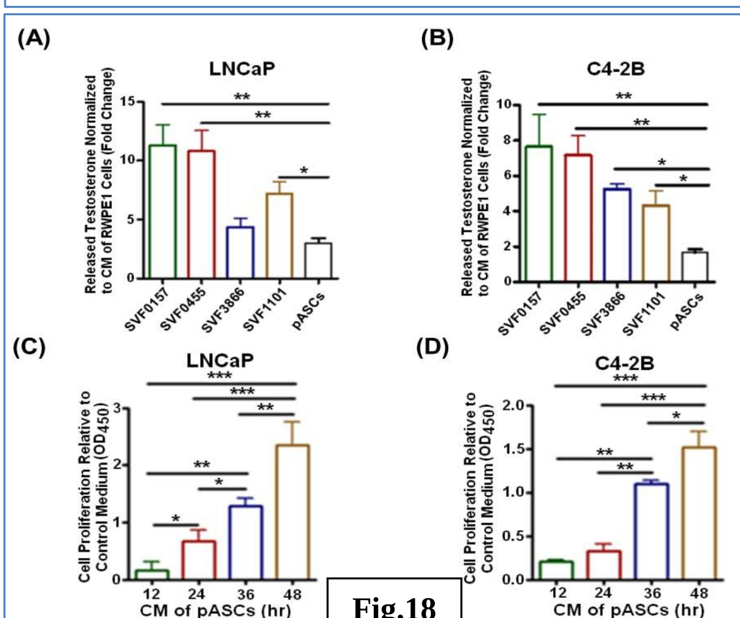


Fig.18

(15). Androgens produced by pASCs stimulate growth of PC cells in vitro and in vivo. We examined the ability of tumor-tropic ASCs to trigger PC cell growth *in vitro* and then *in vivo*. For the in vitro studies (**Fig. 18)**, PCa cells were cultured in presence of CM (50%) from unstimulated or PCa-stimulated ASC^{pc} cells (SVF0157) and monitored the growth of PCa cells for 48 hr. As shown Fig. 18C (LNCaP) and Fig. 18D (C4-2B), PCa cells were able to prime the ASCs to release factors which stimulated the growth of both PCa cell lines, in comparison to CM from unstimulated ASC^{pc} cells. A similar trend of mitogenic response was triggered by the PCa

stimulated SVF0157 in a concentration dependent manner. Comparable results of PCa cell growth induction were also observed by three additional stimulated ASC^{pc} isolates (SVF0455, SVF0366 and SVF1101).

Data are expressed as fold change ± SE after normalization to nASCs (n=3). In (B), the immunoblot analysis of AR, PSA and AME in two representative ASC^{pc} cells (designated SVF0157 and SVF0455) exposed to CM of C4-2B, LNCaP or RWPE1 cells, are shown. In (C) & (D), gene expression of DHT converting enzyme 5-alpha-reductase isoforms (SRD5A1 and 2) in four representative ASCs preincubated with CM of PCa or control cells for 24 hr., are shown. Data are expressed as Mean ± SE in triplicate measurements. *, ** and *** denotes significance at $p<0.05$, $p<0.01$ and $p<0.001$ relative to CM of RWPE1 cells. The results showed significant upregulation of steroidogenic gene transcripts in ASC^{pc} cells which were exposed to CM from the prostate cancer cells, LNCaP or C4-2B cells, as opposed CM from non-tumorigenic RWPE1 cells.

We also observed that exposure of ASCs to CM from PCa cells enabled the release of testosterone (androgen) in media, which was quantified in triplicates by an EIA kit and expressed as Mean ± SE relative to the CM of ASC^{n} cells after normalization to CM of RWPE1 cells (**Fig.18, A & B**). In **C** & **D,** time course assay of LNCaP and C4-2B cell proliferation, respectively, cultured in 50% CM of stimulated pASCs containing 5% charcoal-stripped FBS for 48 hr. Cell proliferation was measured in quadruplicates by WST-1 kit (OD 450) after normalization to DMEM/F12K complete medium. *, ** and *** denotes significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, relative to CM of RWPE1 cells (n=3).

In another set of experiments, PCa cells were cultured in CM of ASC^{pc}s in absence or presence of the antiandrogen bicaultamide, in order to examine if the growth stimulatory effects of ASCs are mediated through hormone production. We observed a significant growth inhibition by bicalutamide, in both LNCaP (androgen-dependent) and C4-2B (androgen independent and hormone sensitive) cells which were cultured under various concentrations of CM from ASC^{pc} cells. [Data not shown] (*Manuscript in Preparation*).

(16). Tumor engrafted ASCs express prostate cancer markers and augment PCa growth *in vivo*. Due to their developmental plasticity and ability to express prostate tumor markers *in vivo*, we sought to examine whether ASC^{ps} pre-stimulated with PC cells alone undergo neoplastic transdifferentiation **(Fig. 19; next page)**. To this end, the tumor-tropic ASC^{pc}s and ASC^{n}s were exposed to the serum-free CM of C4-2B, PC-3, or RWPE1 cells for 72 hr, mixed in Matrigel and then transplanted subcutaneously into the left (PC cells) and right flanks (RWPE1) of each mouse, respectively. Additional control mice were transplanted with unstimulated ASC^{pc}s and ASC^{n}s, C4-2B or RWPE1 cells.

Subcutaneous nodular formation was monitored for 10 weeks. Interestingly, like C4-2B cells, readily visualized lesions were also observed in mice transplanted with pASCs stimulated with CM of PC cells, but not with RWPE1 cells, in 11 out 15 mice studied **(Fig. 19A).** Nodular formation was not observed with parental or PC cells stimulated nASCs (*n=4*). Consistent with PC tumors, histological analysis based on H&E revealed cells with nucleoli and capillary formation (**Fig. 19B**). The PCa induced proliferative responses of ASC^{pc} cells observed *in vitro* were corroborated by *in vivo* experiments in nude mice. The enriched tumor-tropic pASCs were stained with LV-eGFP and were transplanted (subcutaneously) alone or in combination with C4-2B cells. In 5 out of 7 mice, the subcutaneously developed tumors (at 4 weeks) where ASCs were co-transplanted with C4-2B cells were 2 to 3-fold larger than C4-2B tumors alone (**Fig. 19A**). While H&E staining demonstrated consistency with prostate tumors (**Fig. 19B)**, IF analysis demonstrate that only a subset of pASCs engrafted within the C4-2B tumors were found to express AR and PSA *in vivo* (**Fig. 19D**). The tumor-embedded eGFP-stained ASCs were analyzed by H&E and immunofluorescence (IF) analysis for expression of prostate tissue selective markers, and compared with C4-2B alone xenografts. The results suggest that the tumor-tropic pASC not only engrafted and enhanced the tumor mass, but also underwent transdifferentiation into prostate tumor-like cells. The pASCs tumors not only expressed prostate selective epithelial markers (AR, PSA), but also steroidogenic genes (not shown). Additionally, vasculogenesis in the pASC tumors was marked by expression of von Willebrand Factor, and α-smooth muscle actin, selective markers for blood vessel endothelial cells (not shown). Systemically injected ASCs (GFP-labeled) were able to localize to the tumor xenografts. The tumor-tropic ASCs engrafted in PCa tumors and increased tumor growth *in vivo*. The tumor engrafted ASCs augmented PCa growth in vivo and were themselves found to express AR and PSA as well as prostate cancer specific markers such as cytokeratins (5/8) and AMCAR.

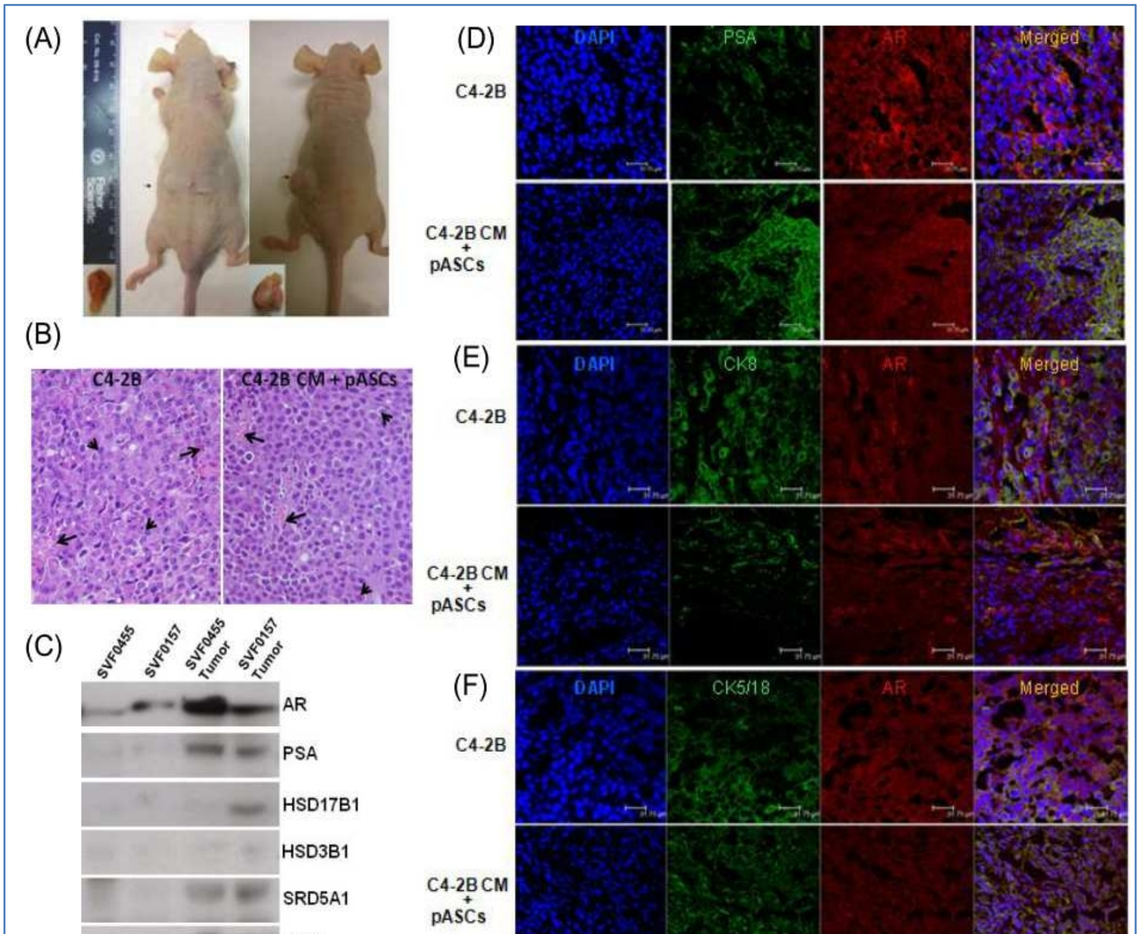


Fig.19

A, representative tumor formation by C4-2B cell CM-simulated (left flask) and RWPE1 stimulated (right flank) ASC^{p}s in nude mice 8 to 10 weeks after transplantation. **B**, Hematoxylin/Eosin stained tumor tissue sections (x *20*) of C4-2B and C4-2B cells' CM-stimulated pASCs. Prominent nucleoli (*arrowhead*) and thin capillaries (*arrow*) are present and no tumor necrosis was observed. **C,** immunoblot analysis of PC epithelial markers and AME in parental and ASC^{p}s tumors SVF0455 and SVF0157. **D**, confocal microscopic analysis of in PC markers in immunofluorescent stained tumor sections of C4-2B and C4-2B CM-stimulated ASC^{p} xenografts. AR stained with red fluorescence whereas PSA, CK8, CK5/18 stained with green fluorescence by individual channels and as merged channels with AR. A strong heterogeneous staining is observed for AR, PSA, and CK5/18 in both xenografts. However, CK8 moderately stained pASC xenografts compared C4-2B tumors. DAPI stains nuclei (blue). Marked expression of PCa selective markers, including CK8 (**Fig. 19E**), CK5/18 (**Fig. 9F**) was also discernible in ASC derived tumors, suggesting mesenchymal-epithelial transition (MET) by ASC^{p} neoplastic cells**.** Scale bars: 50 µm.

Key Research Accomplishments:

- In several well characterized ASC batches, our *in vitro* studies showed that only a small subpopulation of these cells are preferentially attracted towards PCa derived factors.
- Simple 'migration' and 'matrigel invasion' assays were able to demonstrate that *in vitro* enriched clones of ASCs have differential attraction towards tumor-derived factors, especially from aggressive PCa cells.
- *In vitro* stimulation with either TNF-α or IL-1β increased the percentage of this migratory phenotype even in the unfractionated ASCs. ASCs stimulation (TNF-α or IL-1β) increased their endothelial adhesion and tumor-site migratory phenotype, suggesting that patient derived ASCs may have a more activated phenotype and surface markers expressed on these cells enable their higher tumor predilection.
- Certain ASCs possess higher migration and invasion ability towards tumor derived factors, however, serum deprivation of ASCs only enhanced their migration towards growth factors and not towards CM.
- In ASCs enriched for their invasive abilities, significantly increased expression of several adhesion and migration associated genes, e.g. Cdh-2, Cdh-6, Int-α7, HGF, IGF-1, TNFSF10 and FGFR4, and MMP-10 and MMP-11, were observed.
- Coexposure to tumor derived factors (C4-2B CM) enhanced both adipogenic and osteogenic differentiation of AT-MSCs.
- The *in vitro* enriched clones of ASCs (migratory subpopulation) showed significantly increased adipogenic differentiation, and lineage specific transcription factor (PPAR-γ, RUNX and C/EBP-α) expression.
- *In vitro* studies comparing the migration capacity of ASCs from both normal individuals (ASC^{n}) and from PCa-patients (ASC^{pc}), both African Americans and Caucasian Americans (ASC^{AA} and ASC^{CA}), showed differential migratory ability towards PCa tumors.
- ASC migration occurred more rapidly towards Prostate cancer (PCa) cells seeded within bone marrow stromal (BMSC) cultures, suggesting the role of stroma-derived factors and conditioned medium from PCa-BMSC cocultures were seen to produce high quantities of SDF-1α.
- Stimulation of ASCs enhanced the surface expression of CXCR4 and other migration associated genes, e.g. integrins, CAMs and MMPs, which were constitutively higher in the patient-derived ASCs.
- Pre-incubation of ASCs with the CXCR4 antagonist, AMD-3100 inhibited migration potential, suggesting the crucial role of SDF-1α/CXCR4 chemokine axis in ASC recruitment.
- The LV-transduced ASCs showed significantly lower proliferative potential and differentiation capacitites; therefore, a transient transfection system (Neon^{TM}) was optimized to express CXCR4 or HSV-TK genes, which showed good transgene expression and no significant effects on their proliferative potential and differentiation capacities.
- As compared to untransfected ASCs, cells overexpressing CXCR4 showed higher *in vitro* migration towards both tumor-derived factors, as well as towards tumor-associated stroma.
- ASCs expressing HSV-TK demonstrated very potent GCV-induced cytotoxicity in cocultured PCa cells, and *bystander killing* of PCa cells was dependent upon both GCV dose and ASC number.
- Systemically injected ASCs (GFP-labeled) were able to localize to the tumor xenografts. However, the tumor-tropism was much lower with the ASC^{n} cells as compared to the ASC^{pc} cells.
- The tumor-tropic ASCs, enriched from ASC^{pc} cells engrafted in PCa tumors and promoted tumor cell proliferation *in vitro* and increased tumor growth *in vivo*.
- The tumor-tropic ASCs showed increased androgen-metabolizing enzymes (AME) expression and secreted high levels of testosterone *in vitro*, which was, at least partially, responsible for their tumor-promoting activity.
- The tumor engrafted ASCs augmented PCa growth *in vivo* and were themselves found to express AR and PSA as well as prostate cancer specific markers such as cytokeratins (5/8) and AMCAR.
- Strategies that take advantage of enrichment of tumor-tropic ASCs from the non-tumorigenic normal ASC isolates, will be of great advantage towards their use as anti-cancer gene delivery vehicles.

Reportable Outcomes:

Abstracts

1. **Debasis Mondal**, Geetika Chakravarty, Yijun Yang, Marxa L. Figueiredo and Asim B. Abdel-Mageed. *Optimizing the use of adipose tissue-derived mesenchymal stem cells (AT-MSCs) as highly effective anti-cancer gene delivery vehicles.* **2011**. IMPaCT Conference, Orlando, Fl.

2. Robert Davis, Geetika Chakravarty & **Debasis Mondal**. *Enhancement of ganciclovir cytotoxicity with adipose stem cell driven prodrug therapy for prostate cancer.* **2011**. Tulane Health Science Research Day, New Orleans, La.

3. Yijun Yang, Zakaria Y. Abd Elmageed, Raju Thomas, Krishnarao Moparty, **Debasis Mondal**, Krzysztof Moroz, Sudesh Srivastav, Oliver Sartor, and Asim B. Abdel-Mageed. *Prostate tumor secreted factors induce tumor development in patient derived adipose stem cells: Challenging the concept of neoplastic transformation.* **2011**. LCRC Annual Meeting, New Orleans, La.

Manuscripts

1. Geetika Chakravarty, Robert Davis,Yijun Yang, Asim B. Abdel-Mageed and **Debasis Mondal**. Optimizing the use of adipose tissue-derived stem cells (ASCs) as highly effective anti-cancer gene delivery vehicles. *Manuscript In Preparation.*

2. Yijun Yang, Zakaria Y. Abd Elmageed, Raju Thomas, Krishnarao Moparty, Debasis Mondal, Krzysztof Moroz, Sudesh Srivastav, Oliver Sartor, and Asim B. Abdel-Mageed. Prostate tumor secreted factors induce tumor development in patient derived adipose stem cells: Challenging the concept of neoplastic transformation. *Submitted to Science.*

Patents

Provisional Patent filed (# 61/516,671).
Enhancement of Ganciclovir Cytotoxicity with Genetically Engineered Adipose Stem Cell Driven Prodrug Therapy for Cancer.

Grants

New Funding:

1. DoD; Qualified Collaborator Award*: PC102056.* (09/23/2011 – 09/22/2014)

   Stem-Cell Based Therapeutic Targeting of Prostate Cancer Residual Androgens in African Americans
   (Abdel-Mageed; PI)(Mondal; Qualified Collaborator)

2. NIH (NCI); U01: PAR-09-161 (10/01/2011- 09/30/2016)

   Estrogen-ERbeta Axis in Health Disparity of Prostate Cancer
   (Abdel-Mageed; PI)(Mondal; Co-I).

Conclusions:

Our studies demonstrate that although the patient-derived (autologous) ASCs were found to have higher tumor-tropism, possibly due to increased utilization of the SDF-1/CXCR4 chemokine axis, a direct tumor-promoting role for the ASC^{pc}, possibly due to increase in the expression of AMEs and testosterone release, was observed. This will significantly deter the utility of patient-derived (autologous) ASCs as gene-delivery vehicles. We postulate that this will enable the identification of ASCs which should not be used for the delivery of anti-cancer genes.

Our findings further demonstrated that, although ASCs from normal individuals (heterologous) do not possess a high tumor-homing potential, this migratory phenotype can be induced in ASC^{n} cells via their *in vitro* stimulation. A clear advantage of using the ASC^{n} cells was that they did not show any tumor-promoting effect. Gene expression and surface marker analysis showed that specific genes, especially certain cadherins, integrins, MMPs and chemokine receptors, are upregulated in the migratory ASCs. Therefore, we further postulate that strategies which can take advantage of enrichment of tumor-tropic ASCs from the non-tumorigenic ASCs, will be of great advantage towards their use as anti-cancer gene delivery vehicles.

Our preliminary *in vitro* findings with the HSV-TK expression vectors indicated that this 'suicide gene' may be used. However, since the ASC^{pc} cells showed significant increase in tumor growth, they could not be used for the *in vivo* HSV-TK delivery studies. In the future, if we are successful in enriching the tumor-tropic ASCs from normal donors and show that these enriched (tumor-tropic) cells do not cause tumor promotion, our goal would be to use these subpopulation of ASC^{n} cells to deliver the suicide therapy. Since our findings with the ASC^{pc} cells, especially their differential tumor-promoting effects as compared to the ASC^{n} cells, we carried out significant amount of studies to delineate the mechanism/s via which the ASC^{pc} cells may enable increased tumor growth *in vivo*, so strategies to target this phenotype in tumor-recruited stem cells can be developed as a novel anti-tumor approach.

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